Aerosol Delivery of Muramyl Dipeptide to Rodent Lungs

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ABSTRACT Tuberculosis is the single most serious infectious disease worldwide. The respiratory tract is the primary site of infection by *Mycobacterium tuberculosis* (MTB). A number of immunogenic components of the cell wall of MTB, if delivered to the lungs as aerosols, can be used to study the local immune response. The site of deposition of these aerosols can be employed to control their residence time in the lungs. Muramyl dipeptide (MDP) aerosols were delivered to alveolar macrophages in the lungs of rodents. Guinea pig macrophages harvested by bronchoalveolar lavage were examined by differential interference contrast microscopy for morphological changes indicative of activation. Bronchoalveolar lavage fluid was analyzed for the presence of alkaline phosphatase, lactate dehydrogenase, N-acetyl-glucosaminidase (NAG), and total protein content. Rat alveolar macrophages were studied for the production of nitric oxide, by induction of nitric oxide synthase. Twenty-four hours following exposure to an aerosol of MDP, alveolar macrophages exhibited morphological characteristics (spreading and pseudopodia), enzyme activity (NAG 50% above control), and production of the reactive intermediate nitric oxide. Rat macrophages subjected to aerosol exposure to MDP when challenged with a second dose of MDP or lipopolysaccharide exhibited a linear dose response as measured by nitric oxide production. These studies indicate that the topical delivery of an MTB bacterial cell wall component, muramyl dipeptide, results in activation of alveolar macrophages. This approach may be useful in elucidating elements of the immune response to MTB.

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INTRODUCTION

One third of the world's population is infected with *Mycobacterium tuberculosis* (MTB). Of the 8 million individuals manifesting the disease in the last year, almost 3 million have died. The lung is the primary site of infection by this microorganism and the major route of entry for dissemination to the remote tissues (1). Pulmonary tuberculosis is a chronic, slowly advancing disease against which cell-mediated immunity, rather than humoral immunity, is the major defense mechanism (2). Understanding the influence of MTB-derived immunogenic molecules on local elements of the cell-mediated response may elucidate key elements of the initial stages of infection.

Alveolar macrophages (AMs) are the primary cells involved in cell-mediated immunity of the lung. Macrophages become activated in the presence of immunomodulators. These biologically derived and active agents increase the intrinsic host defense to pathogenic microorganisms. AMs are phagocytic cells that secrete enzymes, cytokines, and reactive chemical intermediates to promote removal of particulate matter and invading microorganisms from the lung. AMs are an important element of the initial immune response in the lungs and an attractive aerosol delivery target because they comprise 95% of the nonadherent cells in the lung and possess cell-surface receptors for the known immunostimulants, muramyl dipeptide (MDP) and lipopolysaccharide (LPS).

Muramyl peptides [derived from Bacille Calmette-Guerin (3)], components of the MTB bacterial cell wall, have been shown to induce an immune response following uptake by peritoneal macrophages (4).

These components of the bacterial cell wall have been delivered to macrophages in sites other than the lungs by incorporation in liposomes (5-8). Deposition in the lower lungs depends on particle size and is achieved optimally by particles in the 1- to 5-μm particle size range (9). The challenges of delivering these agents to the lung include factors influencing residence time such as enzymatic degradation and mucociliary transport, both of which remove deposited materials from the lungs (10). Inhalation of aerosols provides a noninvasive delivery system that physically targets the lung as the desired site of pharmacological effect. In the present studies, the intention was to study the local immune response elicited by a specific bacterial cell wall component. In general, localized delivery minimizes systemic exposure and avoids first-pass metabolism by the liver.

MATERIALS

MDP (N-acetylmuramyl-L-alanyl-p-isoglutamine, Adjuvant Peptide, 98% peptide, Sigma, St. Louis, MO) is a nonpyrogenic component of bacterial cell walls. It enhances both humoral and cellular immunity and stimulates macrophage cytotoxicity in vitro. MDP is not metabolized but is rapidly eliminated in vivo through nephritic clearance ($t_{1/2}$ < 20 minutes). LPS from *Escherichia coli* (Serotype 055:B5, Sigma) is an endotoxin derived from bacterial cell walls. Disodium fluorescein (ICN Biomedical, Aurora, OH) was employed as a marker substance to evaluate dose delivered to the animals.

Various cofactors, including β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, N-1630, Sigma); flavin adenine dinucleotide (FAD, F-6625, Sigma); and (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH4, 153622, ICN Biomedicals), were employed to study induction of nitric oxide synthase (iNOS) activity in guinea pigs.

METHODS

Animals were maintained at 72°F on a 12-hour light/12-hour dark cycle and provided food and water ad libitum in accordance with NIH guidelines and University of North Carolina Institutional Animal Care and User Committee protocol #95-264-0-B.

Aerosol Generation and Delivery

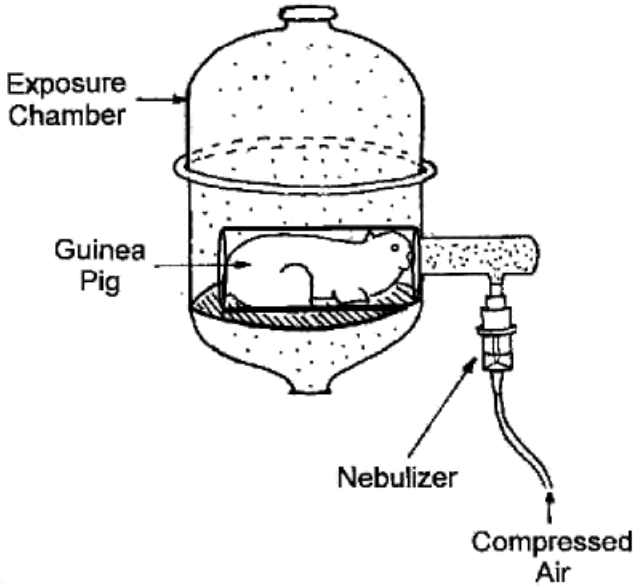

Figure 1: Schematic diagram of the aerosol delivery system and exposure chamber.

A nebulizer (Acorn II; Marquest Medical, Englewood, CO) was used to deliver MDP solution (100 μg/mL in 0.9% w/v saline) at an air pressure of

50 psig into a rodent-modified whole-body ("nose-only") exposure chamber, as shown in Figure 1 (11). Animals were restrained in polypropylene tubes to direct aerosols primarily to the nose in a glass whole-body exposure chamber. Guinea pigs (Dunkin Hartley, 325-375 g; Charles River, Wilmington, MA) or rats (Sprague Dawley, 225-275 g; Charles River) were exposed to an aerosol for a period of 10 minutes (5 mL of solution). Aerodynamic droplet sizes were estimated by inertial impaction (Mark II, Eight Stage Non-Viable; Graseby-Andersen, Smyrna, GA) from the base of the exposure chamber (Figure 1).

Dose Delivery

It was impractical to develop methods for analyzing the dose of immunomodulator administered. However, because nebulizer performance and animal breathing characteristics are well documented, estimates of the dose delivered could be derived. In addition, by using an easily detectable marker for the nebulizer droplets, the dose deposited could be estimated practically.

Estimated doses, of 10 to 20 μg of MDP per animal, were based on breathing frequency (90 breaths/minute), tidal volume (1.8 mL) (12), and aerosol concentration. This estimated dose was in the range likely to activate macrophages, considering its dispersion on the peripheral lung surface, determined by referring to the published literature (13). A group of animals (n = 5) were subjected to a nebulizer study using disodium fluorescein (100 μg/mL in 0.9% w/v saline) solution delivered from a 5-mL reservoir over a 10-minute delivery period. The Acorn II nebulizer was operated at 50 psig. Blood and urine samples were taken from which the plasma concentration versus time and total dose delivered could be estimated. Assays were conducted in glycine buffer, pH 9, by spectrofluorimetry (Perkin Elmer LS50B, Norwalk, CT) at an excitation wavelength of 516 nm and an emission wavelength of 486 nm. The estimated dose delivered was 10.62 ± 0.46 μg of disodium fluorescein.

Broncho-Alveolar Lavage

Animals were killed at specified survival intervals after aerosol exposure. Guinea pigs were anesthetized with ketamine/xylazine/acepromazine (25:12.5:1.25 mg/kg), and the trachea was isolated by a midline incision. Animals were tracheostomized, and a 14-gauge stainless steel catheter was secured with a suture. The animals were exsanguinated, and the lungs were gently perfused 3 times with a single volume (28 mL/kg) of normal saline at room temperature (14). Recovery volume was typically ≥85%. Broncho-alveolar lavage (BAL) fluid was centrifuged and the pellet of cells was recovered. A number of macrophage nonspecific phagocytosis assays were conducted following BAL fluid collection (15). The saline supernatant was assayed for protein content and enzymatic markers of macrophage activation. Resuspended cells were counted, plated on microscope cover glasses, incubated for 1 hour, and fixed and examined for altered morphology using differential interference contrast microscopy. A number of slides were treated in vitro with MDP (100 μL, 100 μg/mL) during incubation. Cell types recovered from the BAL fluid were identified microscopically as an indication of cell infiltration.

Cell Culture

BAL fluid was centrifuged at 500*g* for 5 minutes (GS-15R Centrifuge; Beckman, Palo Alto, CA), and cells were recovered as a pellet. Cells were resuspended in RPMI 1640 media supplemented with 10% heat inactivated fetal calf serum, and 2 mmol/L glutamine. Total cell numbers were counted in a hemacytometer and were adjusted to a cell density of 2×10^6/mL.

Cell suspensions (100 μL) were plated onto standard microscope slides and incubated for 1 hour at 37°C, 95% RH. Some slides were treated in vitro with MDP (100 μL, 100 μg/mL) prior to incubation. Slides were rinsed in normal saline to remove nonadherent cells and fixed with 1% paraformaldehyde. Adherent cells were primarily macrophages (>95%) as determined by a differential staining technique (Camco Stain

Pak; Cambridge Diagnostic Products, Ft. Lauderdale, FL).

Fixed slides were mounted with cover slips and examined by light microscopy (Nikon FXA Research Light Microscope, Garden City, NJ) using differential interference contrast imaging. Cells were visually evaluated for altered morphology and were scored as activated if they exhibited increased cytoplasmic area, visible pseudopodia, or elongated appearance relative to controls.

Nitric Oxide Synthesis

Resident alveolar macrophages were harvested from untreated guinea pigs by BAL, resuspended in supplemented RPMI media, and seeded in 96-well culture plates (2×10^5 cells/well). Cells were incubated for 1 hour at 37°C, 95% RH, 10% CO_2 to allow adherence and then gently rinsed twice with culture media to remove any nonadherent cells. Elicited macrophages were obtained by pretreating animals with MDP aerosols 24 hours prior to BAL and macrophage harvest. This process increased the cell counts and allowed evaluation of the influence of in vivo activation on NO production. Cells were treated in vitro with saline or serial dilutions of the immunostimulants MDP or LPS (0.001 ng/mL-100 μg/mL), with a final well volume of 200 μL. Various cofactors were added to some wells, including NADPH (1 mmol/L), FAD (10 mmol/L), $CaCl_2$ (2 mmol/L), BH4 (10 or 100μM), and mixtures thereof. Cells were incubated for various time periods, usually 24 hours, under the above conditions. Final cell viability was assessed by the crystal-violet methanol method (16) and was typically ≥90%. Nitric oxide (NO) in NO_2 production was evaluated by incubating 100 μL of cell media supernatant with 100 μL Griess reagent (0.05% napthylethylenediamine, 0.5% sulfanilamide, 2.5% phosphoric acid) for 15 minutes at room temperature. Absorbance was measured in a microplate reader (Molecular Device, Sunnyvale, CA) at 562 nm, and samples were quantitated against sodium nitrite standards in distilled water.

BIOCHEMICAL ASSAYS

Alkaline Phosphatase

Alkaline phosphatase (AP) is a lysosomal enzyme indicative of tissue damage. AP catalyzes the reduction of p-nitrophenol phosphate to p-nitrophenol and phosphoric acid in the presence of magnesium ions and adenosine monophosphate. The rate of change in absorbance at 400 nm is directly proportional to AP activity in the sample.

Lactate Dehydrogenase

An increase in the cytosolic enzyme lactate dehydrogenase (LDH) in the bronchoalveolar lavage fluid is indicative of cell damage and lysis. A commercially prepared kit and controls (Accutrol Normal, Sigma) were employed in this assay. LDH catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). Formation of reduced NAD (NADH) results in an increase in absorbance at 340 nm. The rate of change in absorbance at 340 nm is directly proportional to LDH activity in the sample.

N-acetyl-β-D-glucosaminidase

N-acetyl-β-D-glucosaminidase (NAG) is an enzyme secreted by alveolar macrophages during phagocytosis of particulate material. A commercially available kit was employed to assay for this enzyme (Boehringer Mannheim, Indianapolis, IN). 3-Cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide is hydrolyzed by NAG with the release of 3-cresolsulfonphthalein sodium salt (3-cresol purple), which is measured spectrophotometrically at 580 nm.

Total Protein

Increases in total protein are suggestive of transudation of plasma protein (ie, alterations in the alveolar:capillary barrier). Total protein was determined using Pierce Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Sample protein concentrations were determined from a standard curve using bovine serum albumin standards (Sigma). The assay was based on the absorbance shift from 465 to 595 nm that occurs when Coomassie Blue G-250 binds to proteins in an acidic solution.

Reactive Nitrogen Intermediates

NO is produced by induction of iNOS in activated alveolar macrophages as a chemical defense (17,18). NO degrades rapidly to nitrite in solution, which can be colorimetrically assayed (Greiss Reagent). Rats (n = 5) were treated with saline or MDP aerosols. AMs were harvested 24 hours after exposure and cultured in 96-well plates (n = 3/animal) in the presence or absence of a secondary in vitro challenge of MDP or LPS. LPS was selected as a positive control and NO_2 was then measured in the cell supernatant. Student's *t* test (unpaired, 1-tailed) was conducted on mean values.

RESULTS

Aerosols with mass median aerodynamic diameters of 2 μm and geometric standard deviations of 2 were delivered to rodents. Theoretical and practical studies of the dose of MDP delivered were approximately equivalent (10-20 μg). It should be noted that this dose is based on delivery to the entire respiratory tract. Caution should be exercised in extrapolating from this dose to that depositing in the periphery of the lungs.

The modification to the whole-body exposure chamber rendered it effectively a "nose-only" chamber because the animal was constrained and unable to groom; consequently, it was unable to ingest an oral dose of the deposited aerosol droplets.

Guinea pig AMs exhibited altered morphology indicative of activation after MDP aerosol exposure. These effects were time and dose dependent. Enzymatic markers of activation were also increased in BAL fluids after exposure. Cellular influx to the lung was minimal in the period during which animals were studied. MDP stimulated rat AM NO production both in vitro and in vivo.

Cells from saline aerosol-treated control animals were typically rounded in appearance, as indicated in Figure 2. The same cells treated in vitro for 1 hour with MDP exhibit morphological indicators of activation, such as the extension of pseudopodia and oblate morphology.

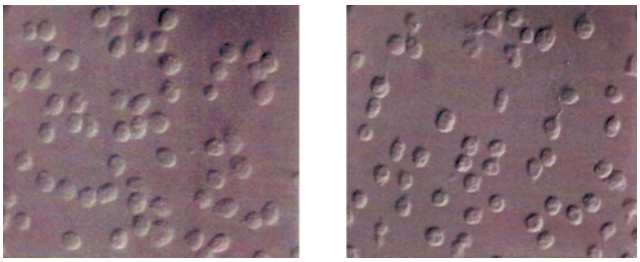

Figure 2: Photomicrographs of guinea pig alveolar macrophages (a) 24 hours following exposure to a saline aerosol and; (b) the same cells (different field) treated for 1 hour with MDP *in vitro*.

Figure 3 depicts cells from animals treated with MDP in aerosol form; numerous activated cells with pseudopodia and oblate morphology are evident 24 hours after exposure. Additional in vitro treatment with MDP increased the percentage of activated cells, and many showed an increase in cytoplasmic area.

Figure 3: Photomicrographs of guinea pig alveolar macrophages (a) 24 hours following exposure to an aqueous MDP aerosol and; (b) the same cell population (different field) treated for 1 hour with MDP *in vitro*.

Animals treated with MDP in aerosol form showed fewer activated cells 48 hours after exposure than at 24 hours after exposure, as illustrated in Figure 4. However, cells remain primed to respond because in vitro treatment with MDP increased the percentage of activated cells with respect to the control values.

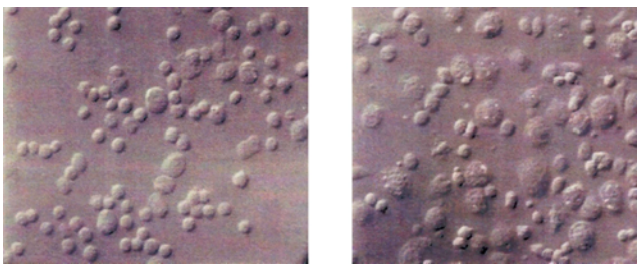

Figure 4: Photomicrographs of guinea pig alveolar macrophages at (a) 48 hours following exposure to an aqueous MDP aerosol and; (b) the same cell population (different field) treated for 1 hour with MDP *in vitro*.

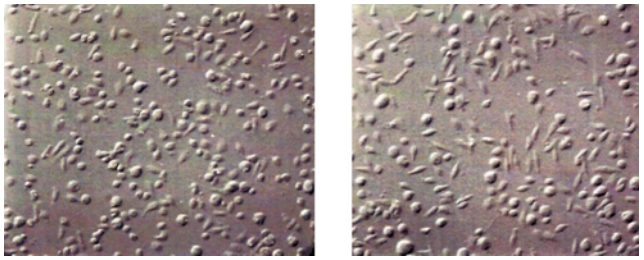

Figure 5: Photomicrographs of guinea pig alveolar macrophages (a) exposed twice at intervals of 24 hours to an aqueous MDP aerosol and (b) the same cell population (different field) treated for 1 hour with MDP *in vitro*.

Cells from animals treated with MDP aerosols on 2 consecutive days show a large number of activated cells at 48 hours. Subsequent in vitro treatment with MDP has little additional effect. Figure 5 illustrates these phenomena.

Table 1 illustrates the enzyme activity and cell infiltrate following various muramyl dipeptide treatments. Neutrophil, basophil, and eosinophil numbers did not change following exposure. The number of lymphocytes tripled up to 72 hours, whereas monocyte and macrophage numbers peaked at 5- to 8-fold higher than untreated control at 24 to 48 hours, returning to baseline at 72 hours.

TABLE I: Cell influx and enzyme levels retrieved from guinea pig bronchoalveolar lavage fluid.

Time Following Aerosol Exposure (hours)	Total Cell Number ($x10^6$)	N-Acetyl Glucosaminid-ase (% above Control)	Alkaline Phosphatase (% above control)	Total Protein (% above control)
0[a]	1.90	-	-	-
6	6.11	-15.4	14.9	-14.6
19	9.18	29.3	23.4	74.0
24	8.57	58.0	44.7	74.0
48	7.07	37.9	48.9	78.0
48[b]	15.40	196	74.5	115.6

a. Saline treated control animals

b. Two in vivo exposures on successive days.

TABLE II: Qualitative summary of the morphological changes (by number) of the guinea pig alveolar macrophage populations observed: - < 10% spreading; ± 10-20% spreading, some tvisible pseudopodia; + 20-35% spreading, numerous cells with pseudopodia or elongated morphology; ++ >35% spreading or altered morphology

Time Following Aerosol Exposure (hours)	Aerosol Exposure to MDP	Aerosol exposure followed by in vitro exposure to MDP
0[a]	-	±
6	±	±
19	+	++
24	+	++
48	±	+
48[b]	++	++

a. Saline treated control animals.

b. Two in vivo exposures on successive days

Lactate dehydrogenase activity did not change in respect to control values in any of the studies. The remaining enzyme levels and total protein increased at 24 to 48 hours in respect to controls, as shown in Table 1. The increase in NAG, by 58% at 24 hours, is indicative of the phagocytic activity of the macrophages. The increase in alkaline phosphatase, by 44.7% at 24 hours, represents a nonspecific local inflammatory response. The increase in total protein, by 78% at 48 hours, is indicative of the presence of additional enzymes and mediators resulting from the action of the aerosol. MDP does not contribute to the total protein because it is cleared rapidly. In addition, it can be seen that there was little change in total protein at early time points. Table 2 is a qualitative summary of the morphological changes of the alveolar macrophage populations observed after aerosol and in vitro treatments.

Neither resident nor elicited guinea pig AMs showed detectable production of nitrite under any test conditions. Various in vitro stimulation periods of 6, 12, and 24 hours were examined initially, and all further experiments were standardized to a 24-hour incubation. Both resident and elicited GP macrophages failed to respond to LPS or MDP stimulation in vitro over a wide concentration range. Elicited guinea pig AMs also failed to respond to LPS stimulation in the presence of various cofactors, including NADPH, calcium, FAD, and 2 different concentrations of BH4 (1 mmol/L or 100 mmol/L), either alone or in combination. In contrast, resident rat AMs showed significant nitrite production when stimulated with LPS (0.1, 5, and 10 μg/mL) or MDP (10.0 or 20.0 μg/mL). Therefore, preliminary studies showed that guinea pig AMs did not produce detectable quantities of NO, a reactive intermediate implicated in the immune response. In order to study the production of NO in response to the presence of MDP, a species was selected in which alveolar macrophages are known to exhibit iNOS activity.

Figure 6 shows indirectly the production of NO detected in the form of nitrite (NO_2) by alveolar macrophages lavaged from the lungs of rats following aerosol exposure to MDP, and including a variety of treatments in vitro.

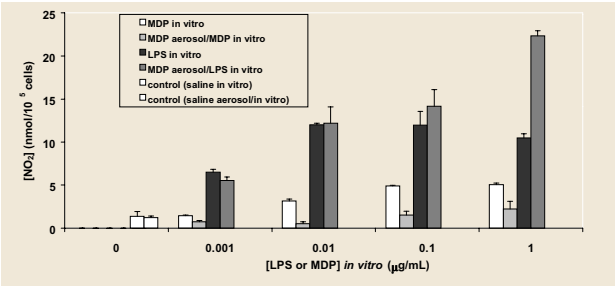

Figure 6: Nitrite concentration (NO_2) reflecting NO production by rat alveolar macrophages in broncho-alveolar lavage fluid under a variety of conditions as determined using Greiss reagent (Mean ± SE, n=15).

Treatment with MDP in vitro produced a linear dose response in the range of 0.001 to 1 μg/mL of 1-5 nmol $NO_2/10^5$ cells. Treatment with LPS in vitro in the same concentration range resulted in approximately the same increase in NO production but over a slightly higher range, 6-12 nmol $NO_2/10^5$ cells. Pretreatment with MDP aerosol reduced the production of NO with the subsequent MDP treatment in vitro (0.5-2 nmol $NO_2/10^5$ cells). MDP aerosol pretreatment had a significant effect on the NO production by cells treated with LPS in vitro (5-22 nmol $NO_2/10^5$ cells over the concentration range 0.001-1 μg/mL LPS). It is clear that aerosol pretreatment primes the macrophage for a subsequent challenge. However multiple exposure to the same immunomodulator may condition the response.

MDP aerosol followed by MDP in vitro resulted in a significant ($P < .05$) decrease in the mean NO production in comparison with the MDP treatment alone in vitro at all concentrations ($P < .001$ for 0.001, 0.01, and 0.1 μg/mL and $P < .025$ for 1 μg/mL). MDP aerosol followed by LPS in vitro resulted in a significant increase in the mean NO production compared to the LPS treatment alone in vitro at 1 μg/mL ($P < .001$) and a significant decrease at 0.001 μg/mL ($P < .05$) but had no effect at 0.01 and 0.1 μg/mL ($P > .05$).

DISCUSSION

Morphological changes were observed in alveolar macrophages following treatment of guinea pigs with aerosols of MDP. These changes were concomitant with increased enzyme activity. The production of N-acetyl-glucosaminidase is an indicator of phagocytic activity and is consistent with the observation that some of the cells observed microscopically produce pseudopodia following MDP treatment. There was also some indication of a nonspecific immune response, as indicated by elevated alkaline phosphatase activity and total protein concentrations in BAL fluid.

These studies indicated that aerosol delivery of peptide immunomodulators directly to rodent lungs resulted in activation of the local cellular immune system. They also demonstrated a “priming” effect of MDP on AMs, as indicated by the enhanced response of cells stimulated in vivo to secondary in vitro challenges.

The advantage of an aerosol delivery approach is that the immunogen is presented in a manner similar to

the microorganism. Consequently, the local effects are of direct relevance to the disease state. In addition, the presence of airway epithelial cells and proximity to the interstitium and vasculature allow the influence on the local environment to be studied.

Control rat AMs showed low basal levels of iNOS activity but responded to in vitro MDP or LPS exposure, as depicted in Figure 6. Cells from animals treated with MDP in aerosol form also showed low basal levels of iNOS activity, but became refractory to a second MDP challenge in vitro. However, their response to an LPS challenge in vitro was enhanced relative to controls treated with saline.

A variety of markers of inflammation or immunity can be employed to evaluate aerosol challenges to the lungs. Cell and enzyme studies have been described. There are no commercially available reagents for guinea pig cytokines/chemokines. However, this species, in common with humans, does not readily exhibit iNOS activity. It remains to be seen whether either species requires specific cofactors to exhibit such a response. This is a subject of great interest to molecular immunologists at present. The rat alveolar macrophages exhibit iNOS activity and were consequently selected for evaluation of the biochemical responses to MDP aerosol delivery. The apparent contradiction of the response of rats, based on nitrite production, and guinea pigs, based on cell morphology, to MDP aerosol followed by MDP in vitro alone may represent a fundamental difference in these species' immunological response to such a challenge. Rats apparently become refractive to a second challenge with the same immunomodulator, whereas guinea pigs exhibit an enhanced response under the same experimental conditions. This difference has implications for the selection of an animal model, in regard to relevance to humans.

Models for human lung diseases in rodents are numerous, and these studies form the basis for a more detailed investigation of the local immunological response. Future work will focus on more specific aspects of the inflammatory response, including further identification of infiltrating cell types, evaluation of cytokine/chemokine production, and cytotoxicity of activated macrophages against bacterial targets. These studies are of particular significance because methods of studying the relationship between acquired immunodeficiency syndrome and serious opportunistic bacterial infections are required.

CONCLUSION

Models for human lung disease in guinea pigs are numerous. Little is known of the immunological activity of the lung at the molecular level in this species. These studies initiate a program to evaluate the guinea pig immune response. Treatment of macrophages with MDP in vitro is known to induce activation. Our preliminary data are indicative of alveolar macrophage activation after aerosol administration in vivo.

Exposed animals exhibit a cellular influx to the site of administration, and the alveolar macrophages exhibit altered morphology relative to controls. Total protein and cytosolic enzyme levels of NAG and alkaline phosphatase are also elevated, indicating a nonspecific immune response. These effects are time-dependent relative to residence time in the lung. Dose dependence is also demonstrated between doubly- and singly-exposed animals.

The basis of conducting aerosol-exposure experiments and evaluating the biological outcome of administering immunomodulators directly to the lungs has been established. Further work is required to investigate the detailed biochemical and immunological responses of alveolar macrophage from different species to activation using immunomodulators.

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REFERENCES

1. Daniel T, Ellner J. Immunology of Tuberculosis. In: Reichman L, Hershfield E, eds. *Tuberculosis.* New York: Marcel Dekker; 1993:75-101.
2. Pelczar M, Chan E, Krieg N. *Microbiology.* New York: McGraw-Hill; 1986:815-818.
3. Lederer E. Recent progress in the study of natural and synthetic immunomodulators derived from bacterial cell walls. In: Yamamura Y, Kotani S, eds. *Immunomodulation by Microbial Products and Related Synthetic Compounds.* Princeton, NJ: Excerpta Medica; 1992:3-16.
4. Burleson G. Pulmonary immunocompetence and pulmonary immunotoxicology. In: Smialowicz R, Holsapple M, eds. *Experimental Immunotoxicology.* Boca Raton, FL: CRC Press; 1995:119-141.
5. Fidler I. Systemic macrophage activation with liposome-entrapped immunomodulators for therapy of cancer metastases. Res in Immunol. 1992;143:199-204.
6. Phillips N, Chedid L, Bernard J, Level M, Lefrancier P. Induction of murine macrophage tumoricidal activity and treatment of experimental pulmonary metastases by liposomes containing lipophilic muramyl dipeptide analogs. J Biol Resp Mod. 1987;6:678-690.
7. Sone S, Tachibana K, Shoho M, Ogushi F, Tsubura E. Potential value of liposomes containing muramyl dipeptide for augmenting the tumoricidal activity of human alveolar macrophages. J Biol Resp Mod. 1984;3:185-194.
8. Phillips N, Moras M, Chedid L, et al. Activation of macrophage cytostatic and cytotoxic activity in vitro by liposomes containing a new lipophilic muramyl peptide derivative, MDP-L-alanyl-cholesterol (MTP-CHOL). J Biol Resp Mod. 1985;4:464-474.
9. Hickey A. Summary of common approaches to pharmaceutical aerosol administration. In: Hickey A, ed. *Pharmaceutical Inhalation Aerosol*
10. *Technology.* New York: Marcel Dekker; 1992:255-288.
11. Altiere R, Thompson D. Physiology and pharmacology of the lung. In: Hickey A, ed. *Inhalation Aerosols: Physical and Biological Basis for Therapy.* New York: Marcel Dekker; 1996:85-137.
12. Hickey A, Suarez S, Bhat M, et al. Efficacy of rifampicin-poly(lactide-co-glycolide) microspheres in treating tuberculosis. In: Dalby R, Byron P, Farr S, eds. *Respiratory Drug Delivery VI.* Buffalo Grove, IL: Interpharm Press; 1998:201-209.
13. Chaffee V. Surgery of laboratory animals. In: Melby E, Altman N, eds. *Handbook of Laboratory Animal Science.* Boca Raton, FL: CRC Press;
14. 1974;263.
15. 13. Seyler I, Morin C, Barratt G, Appel M, Devissaguet J-P, Puisieux F. Induction of macrophage NO-synthase by an immunomodulator entrapped within polymeric nanocapsules. Eur J Pharm Biopharm. 1995;41:49-54.
16. 14. Fleisch J, Armstrong C, Roman C, et al. Recombinant human secretory phospholipase A_2 released thromboxane from guinea pig bronchoalveolar lavage cells: in vitro and ex vivo evaluation of a novel secretory phospholipase A_2 inhibitor. J Pharm Exp Ther. 1996;278:252-257.
17. 15. Neldon D, Lange R, Rosenthal G, Comment C, Burleson G. Macrophage nonspecific phagocytosis assays. In: Burleson G, Dean J, Munson A, eds. *Methods in Immunotoxicology.* Vol. 2. New York: Wiley-Liss; 1995:39-57.
18. 16. Leibovitz A, Stinson I, McComb III W, McCoy C, Mazur K, Mabry N. Classification of human colorectal adenocarcinoma cell lines. Cancer Res. 1976;36:4562-4569.
19. 17. Gibaldi M. What is nitric oxide and why are so many people studying it? J Clin Pharmacol. 1993;33:488-496.
20. 18. Jorens P, Van Overeld F, Bult H, Vermeire P, Herman A. Serine-protease inhibitors modulate nitric oxide-synthase activity of alveolar macrophages. Agents Actions. 1992;36:243-247.
21. 19. Pettis R, Hall I, Hickey A. Effect of muramyl dipeptide aerosols on guinea pig alveolar macrophages. Pharm Res. 1996;13:S166.
22. 20. Pettis R, Hickey A. Aerosol delivery of peptide immuno-modulators to rodent lungs. In: Tam J, Kaumaya P, eds. *Peptides: Frontiers of Peptide Science.* Boston, MA: Kluwer Academic Publishers; 1999:845-846.